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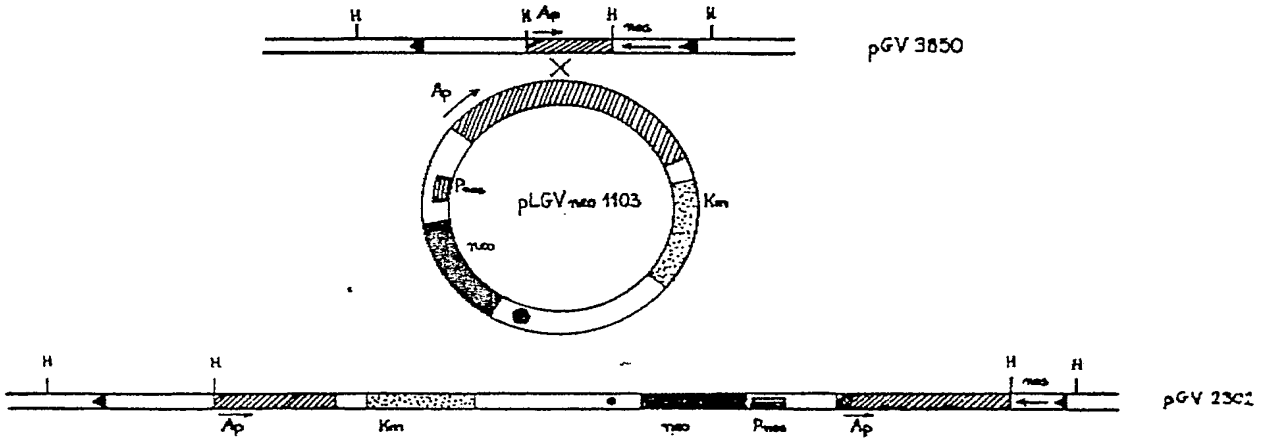

(57) Abstract

Process for preparing genetically stably transformed monocotyledonous plant cells comprising the transformation of monocotyledonous cells using *Agrobacterium* harbouring a hybrid Ti-plasmid vector.

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Title:

PROCESS FOR PREPARING GENETICALLY STABLY TRANSFORMED MONOCOTYLEDONOUS PLANT CELLS

Recombinant DNA techniques have made the genetic engineering of organisms a challenging prospect. The cells of some higher plants exhibit excellent regeneration capacities and therefore are good materials for the genetic engineering of higher organisms.

Furthermore, in higher plants, a system is available to introduce foreign DNA into the plant genome. This system is provided by the tumor inducing plasmid from the Gram$^-$ soil bacterium Agrobacterium tumefaciens. Over the past fifty years it has been clearly shown that Agrobacterium causes neo-plastic transformations called Crown Gall on wounded tissue of an extremely wide range of dicotyledonous plants. A consensus exists in the literature that monocotyledonous plants are not sensitive to tumor induction by Agrobacterium tumefaciens. The molecular basis of the transformation process by Agrobacterium is the stable integration of T-DNA, a well-defined fragment of the Ti-plasmid, in the plantcell genome. In the last years, important progress has been made to facilitate the use of the Ti-plasmid as a vector for plant genetic engineering. Mutational analysis of the Ti-plasmid has shown that expression of T-DNA genes is not required for transfer and integration of T-DNA. Small directly repeated sequences which flank the T-DNA (border sequences) were suggested to

play a key-role in T-DNA integration. This has now been firmly demonstrated since the introduction of a short synthetic DNA, identical to such a border sequence, is sufficient to restore T-DNA integration from a Ti-plasmid that lacks one end of the T-DNA. These observations have allowed to construct non-oncogenic Ti-plasmid vectors from which the tumor genes have been removed by an internal deletion in the T-DNA. These Ti-plasmids still contain the border sequences and consequently transfer T-DNA without tumor induction. An example of such a Ti-plasmid derived vector for plant engineering is pGV 3850. This Ti-derivative has proven very useful. It contains a substitution of the internal T-DNA genes by the commonly used cloning vehicle pBR322. Plant cells transformed by pGV 3850 have the same regenerative capacity as untransformed cells and several procedures have been developped to obtain intact plants which contain the pGV 3850 T-DNA. pGV 3850, through the pBR 322 sequences present in its T-DNA, is an efficient acceptor plasmid for gene transfer experiments in plant cells (see European patent application No. 0 116 718). Indeed, genes cloned in pBR 322-like plasmids are transfered to Agrobacterium and inserted via homologous recombination into the pGV 3850 T-DNA in a single experimental step. Another major advance in the application of T-DNA as a vector is the use of plant regulatory sequences to express chimeric genes in plants. These chimeric genes contain a promotor region derived from a gene which is naturally expressed in plant cells, the sequence to be expressed, and preferentially a 3'-non-translated region containing a poly-adenylation site of a gene which is naturally expressed in plant cells. For example, using the nopaline synthase promotor and bacterial antibiotic resistance genes, dominant selectable markers for plant cells have been constructed. These techniques have now been used in dicotyledonous plants such as Nicotiana tabacum, and Nicotiana plumbaginifolia. Until now, the vast group of monocotyledonous plants was considered as not sensitive towards infection by Agrobacterium. Recently it has been demonstrated that it is very likely that Ti-plasmid genes from Agrobacterium can also be - at least temporarily - transferred and expressed in monocotyledonous plants; see Ref. 1. This was shown by

inoculating plants belonging to the families of the Liliaceae and Amaryllidaceae with Agrobacterium. Although no tumor formation could be observed, investigators could demonstrate the presence of opines in the wound tissue of these plant cells. Opines are specific compounds which are found in tumor cells induced by Agrobacterium. The genes involved in the synthesis of opines are encoded by the T-DNA of Agrobacterium, but the regulatory sequences involved in their expression are of an eucaryotic type. This explains why these genes are expressed in a plant environment. Thus, the observation of the presence of opines in the wound tissue of infected monocotyledonous plants suggested at least a temporarily transfer and expression of Ti-plasmid genes into the monocotyledonous plant.

However, these data did not provide any prove for stable transfer of Ti-plasmid DNA into the plant cell. The investigators did not show the monocot tissue to produce opines after sub-culture under aseptical conditions.

The object of the invention is to provide a process for preparing transformed monocotyledonous plant cells which are genetically stable. A further object of the invention is the preparation of genetically transformed monocotyledonous plant cells as well as plants regenerated therefrom.

The present invention relates to a process for preparing transformed plant cells which are genetically stable comprising the transformation of plant cells using Agrobacterium harbouring a hybrid Ti-plasmid vector, characterized in that the plant cells are derived from a monocotyledonous plant. The invention makes use of hybrid Ti-plasmids such as those described in European Patent Application No. 0116718. These are Ti-plasmids, from which the oncogenic genes or tumor inducing genes have been removed from the T-DNA. Furthermore, according to the present invention, Ti-plasmids can be used which contain a gene of interest operatively linked to a suitable promoter, e.g. the nopaline-synthase-promoter. Such a combination is also called a chimeric gene.

Additionally, these plasmids contain chimeric antibiotic resistance genes, which provide a dominant selectable marker for plant cells.

The data presented here are the first example proving stable transfer of chimeric genes into monocotyledonous plant cells and plants, as well as the expression of the chimeric genes. These results now open the way for the genetic engineering of plants of great agricultural importance. The most important plant species in agriculture on a world-wide basis indeed are monocotyledonous plants.

For many of these plants procedures exist which allow to regenerate intact plants from tissue culture. The process described in this patent application will make it possible to introduce new genetic traits in such plants. Examples of new traits which can have high commercial or agricultural value are resistance to insects, resistance to herbicides, resistance to viruses resistance to fungi, or genes coding for enzymes which will interfere in important biochemical pathways, for example those leading to the synthesis of essential amino-acids. The latter could lead to plants having higher nutritional value.

Fig. 1: Construction of the intermediate plasmid vector pLGVneo1103.

Step 1: Construction of a 290 bp Sau 3A fragment containing the nopaline synthase promoter.

The construction is similar to the one described by Herrera-Estrella et al. (ref. 2).

The starting plasmid is pLGV13 (ref. 2). 20 µg of pLGV13 DNA were digested with BglII and treated with 6 units of Bal31 exonuclease (Biolabs, New England) for 1, 1.30, 2, 2.30 and 3 minutes in 300 µl of 12 mM $MgCl_2$, 12 mM $CaCl_2$, 0.6 M NaCl, 1 mM EDTA and 20 mM tris-HCL, pH 8.0, at 30°C. 1 µg of Bal31-treated molecules of each reaction were ligated at 4° C to 0.13 µg phosphorylated BglII linkers (Biolabs, New England) with 2 units T4 DNA ligase in a

total volume of 20 µl. After the T4 ligase was inactivated at 68° C for 10 minutes, each ligation mix was digested with 20 units BglII for 1 h at 37° C. Subsequently, 50 ng DNA were recircularized with 0.1 unit T4 DNA ligase in a total volume of 100 µl for 20 h at 4° C. One fifth of this ligation mixture was transformed into competent E. coli K 514 cells (ref. 3) as described by Dagert and Ehrlich (ref. 4). Cells were plated on LB medium (ref. 5), supplemented with carbenicillin (100 µg/ml). The deletion end points in the plasmids were first analyzed by measuring the size of the newly generated BclI-BglII fragments of the recombinant plasmids. The nucleotide sequence of the exact deletion end points in some plasmids were determined.

One plasmid revealed the following DNA sequence at the deletion end point:

CATAAATTCCCCTCGGTATCCAATTAGAGTCTCATATTCACTCTCAATCAAAGATCT

5' end of transcript

BglII

From this plasmid the nos promoter can be isolated as a 290 bp BclI-BglII fragment.

Step 2: Insertion of Pnos in front of the neo gene.

All subsequent recombinant DNA manipulation steps use the methods described in the laboratory manual by Maniatis et al. (ref. 6).

The 290 bp BclI-BglII fragment containing the nopaline synthase promoter (Pnos) was purified from a 2 % agarose gel by electroelution and inserted into pKM109-2 (ref. 7).

One of the resulting recombinants, pLGVneo011 contains the Pnos fragment in the orientation such that it will direct transcription of the neomycin phosphotransferase gene (neo) in plant cells. The orientation was determined by SacII-EcoRI digestion.

Step 3: Insertion of the 3' end of the octopine synthase gene behind the neo gene.

A fragment containing the 3' untranslated region of the octopine synthase gene was isolated from pGV99 (ref. 8). This was achieved by digesting 20 µg of pGV99 DNA with 20 units of PvuII for 1 h at 37° C, separation of the fragments on a 0.8 % agarose gel and isolation of the 706 bp PvuII fragment by electroelution. The 706 bp PvuII fragment was inserted into the SmaI site of pLGVneo011. One of the resulting recombinant plasmids, pLGVneo11 contains the PvuII fragment in the correct orientation with respect to the neo gene.

Step 4:

Finally, a SalI fragment from pUC4K (ref. 9) was inserted into the unique SalI site of pLGVneo11 to produce pLGVneo1103.

During this step, two copies of pLGVneo11 were ligated in inverted orientation together with the Km^R fragment from pUC4K. For reasons of simplicity the plasmid pLGVneo1103 as presented in Figure 1 only contains 1 copy of the pLGVneo11 plasmid.

Thus, pLGVneo1103 contains a chimeric neo gene consisting of the nopaline synthase promoter, the coding region of the neo gene and the 3' end of the octopine synthase gene, including the polyadenylation signal. The advantage of this chimeric neo gene over the one previously described (ref. 10) is that the AUG initiation codon for the neomycin phosphotransferase is the first AUG codon in the 5' end of the transcript. This allows optimal translation to occur, and hence to provide resistance levels in plants towards Kanamycin, which are substantially higher than obtained with similar construction where the AUG initiation codon is not the first AUG triplet on the 5' end of the transcript.

Fig. 2: Schematic representation of the T region of pGV3850 and of intermediate vector pLGV1103.
The crossed lines indicate the regions which were involved in co-integration of pGV3850 with pLGVneo1103. The T region of hybrid Ti-plasmid pGV2302 is represented.

Ap,KM : gene encoding resp. ampicillin and kanamycin resistance
Pnos : nopaline synthase promoter
neo : neomycin phosphotransferase
● : 3' end of octopine synthase

Fig. 3: Detection of nopaline in tissue extracts.

The method used for the detection of nopaline is described in examples 1 and 2.

The process of the present invention will now be explained in more detail by the following two examples, in which a specific vector plasmid is used.

These experiments provide the first proof for stable transfer of genetically engineered DNA into a monocotyledonous plant as well as the first proof for stable expression of such DNA into a monocotyledonous plant.

The experiments clearly prove that the techniques developped for genetic engineering in plants as described in European Patent Application No. 0116718 can be used to genetically engineer a monocotyledonous plant species. Our observations now allow to introduce and express economically interesting genes in Asparagus officinalis and also open the way to adapt these techniques for use in other agriculturally important monocotyledonous plants.

Example 1: Isolation of a cell culture line which is derived from a monocotyledonous plant and that contains and expresses T-DNA genes of Agrobacterium tumefaciens.

Plant cells of Asparagus officinalis L.cv. (Ruhm v. Braunschweig) transformed with an oncogenic Ti-plasmid (e.g. C58) were prepared. The procedure involves the following steps:

- in vitro inoculation of stem fragments.
- in vitro culture of the proliferating tissue on hormone free medium.
- detection of nopaline in established axenic cell lines.
- detection of T-DNA in established axenic cell lines.

In more detail the procedure is carried out as follows:

(1) Germinate and grow Asparagus officinalis L.cv. "Ruhm v. Braunschweig" in pots with vermiculite under artificial light (5000 Lux, 16 hrs/day, cool white fluorescent tubes) at 22° C. Water daily with mineral fertilizer solution (Bayfolan Bayer, 0.3 ml/l) supplemented with the micro-elements of the Murashige and Skoog medium (see ref.12).

(2) Remove young spears, 20 cm long from 10 month old seedlings. They are 2-3 mm in diameter.

From now on, all manipulations are done under aseptical conditions.

(3) Surface sterilize the spears applying the following treatment
- 70 % ethanol for 2 min.
- 0.5 % $HgCl_2$ in H_2O for 5 min.
- 10 % commercial bleach and 1 % laboratory detergent (Decontamin) for 30 min.

- five rinses with sterile tap water.

(4) Cut spears in 3 cm long segments with a scalpel.

(5) Place 20 fragments vertically and upside down in a 250 ml glass top jar containing 50 ml half-strength LS medium (Medium containing the mineral components, iron chelate, inositol, thiamine and sucrose of the Linsmaier and Skoog medium (see ref. 13)), solidified with 0.7 % Difco Bacto agar. The fragments are inserted 5 mm in the agar medium.

(6) Grow Agrobacterium tumefaciens strain C58 on an LB plate for 48 hrs. at 28°C. Take bacteria from the plate with a spatula and apply them to the top of the segments. Incubate for 1 month in a plant culture room (1.500 Lux, 16 hrs/day, at 24 ° C)

(7) Excise the proliferating tissue from the upper ends of the stem fragments with a new scalpel blade. The formation of tumorous proliferation on asparagus is quite reproducible, but it is essential to involve a large number of fragments in the infection process since only two to three out of the twenty infected segments routinely reacted with clearly detectable proliferations.

(8) Place the fragments in a 5 cm petridish containing 10 ml hormone-free LS medium supplemented with 200 mg/l glutamine and 500 mg/l Cefotaximum (Claforan, Hoechst). Incubate for one month.

(9) Transfer calli on the same medium in a 5 cm petridish. Incubate for one month.

(10) Remove all necrotic parts of the tissues and transfer on the same medium with a lowered concentration of Claforan (250 mg/l). The transformed cells will grow as a compact, hard callus with a white-yellow color. Its rate of proliferation is slower than for untransformed asparagus tissue growing on the same medium supplemented with 1 mg/l 1-Naphtalene acetic acid and 1 mg/l of 6-Benzylaminopurine. Untransformed asparagus stem explants do not proliferate on hormone-free medium.

(11) Use approx. 20 mg samples of the hormone independant tissue to detect expression of the nopaline synthase gene.
Ground the tissue in an Eppendorf tube with a plastic rod.

(12) Centrifuge for 2 minutes in an Eppendorf centrifuge.

(13) Apply 2 microliter of the supernatant on a 15 cm long Whatman 3 MM paper.

(14) Perform electrophoresis towards the anode for 90 min. at 350 V using as buffer 5 % pyridine and 0,25 % acetic acid in H_2O.

(15) Dry the paper under air current.

(16) Detect nopaline using the procedure of Otten and Schilperoort (ref. 16).

Using this procedure (steps 1 to 10), a tissue culture line which grows vigorously on hormone-free media was obtained. Testing for nopaline in this tissue as described (steps 11 - 16) shows that it indeed produces significant levels of nopaline (see Fig. 3). It was concluded that T-DNA promoters are active in these cells, with an efficiency comparable to that found in dicots. Genes for hormone independent growth are also expressed since the transformed cells proliferate on hormone free culture medium.

The presence of T-DNA in the transformed cell line was further demonstrated by Southern blot analysis of total DNA established from the cell cultures, using cloned T-DNA fragments as radioactive probes. The procedure involves the following steps:

(17) Isolate DNA from the transformed cell lines according to the method of Dellaporta et al (ref 21).

(18) Digest the DNA with different restriction enzymes, perform agarose gelelectrophoresis and transfer the DNA to nylon filters (Gene Screen Plus, ® New England Nuclear) using the protocol provided by the manufacturer.

(19) Hybridize the filters using 50% formamide and 10% dextrane sulphate, as described in the protocol, against ^{32}P radioactive labeled probes (ref 22) which cover the T-DNA.

The most important conclusion out of these data is that we could detect all internal fragments from EcoRI-16 on the left hand side of the T-DNA to BamHI-10 on the right hand side. Within the limits of the accuracy of this procedure, the same T-DNA is integrated in monocots as in dicots.

Using the same procedure, a cell line transformed with the oncogenic octopine type Ti-plasmid pTi B6S3 was obtained. Steps 1-11 are identical except for step 6 where the Agrobacterium strain GV3100 (pTi B6S3) (Holsters et al, 1978, ref 19) is used.

The presence of the opines octopine and agropine in the transformed cell line was demonstrated by paper electrophoresis as described previously (Leemans et al, 1981, ref 20). The presence of the TL and TR segments of the octopine T-DNA in the transformed tissue was demonstrated by Southern blot analysis of total DNA extracted from the cultures.

The procedure used was the same as described in step 17-19.

Example 2: Isolation of monocotyledonous plant cells containing and expressing a chimeric gene.

Plant cells of Asparagus officinalis L. cv. "Ruhm v. Braunschweig" transformed with a non-oncogenic Ti-plasmid derivative (eg. pGV2302) were prepared. The hybrid Ti-plasmid used in this experiment and carrying a gene of interest was pGV2302. This plasmid is a derivative of pGV3850 which is described in European Patent Application No. 0116718 and was obtained as follows. According to the method of Van Haute et al. (ref. 11) helper plasmids were transferred from GJ23 cells to E. coli K514$r^{-}m^{+}$ containing pGLVneo1103 (for detailed description see below). Alternatively pGLVneo1103 can also be transferred to the helper plasmid containing GJ23 cells. Finally, pGLVneo1103 is mobilized during conjugation from the bacteria strain containing the helper plasmids as well as pGLVneo 1103 to Agrobacterium tumefaciens $C_{58}C_1Rif^{R}$pGV3850 which carries the hybrid Ti-plasmid pGV3850 (ref. 18). This plasmid transfer is followed by an in vivo recombination event in the recipient strain of Agrobacterium tumefaciens. The result is the recombinant plasmid pGV2302 which carries the genetic information for rifampycin and kanamycin resistance (see Fig. 2). The strain $C_{58}C_1Rif^{R}$pGV2302 of Agrobacterium tumefaciens carrying plasmid pGV2302 and the strain of E. coli carrying the plasmid pGLVneo1103 were deposited with the Deutsche Sammlung für Mikroorganismen, Göttingen, West Germany, under the accession numbers DSM 3168 and DSM 3169, respectively.

pLGVneo1103 (a detailed description of the construction is given in Fig. 1) is an intermediate vector which contains a chimeric gene consisting of the promoter of the nopaline synthase gene (Depicker et al., ref.14), the coding region of the neomycin phosphotransferase gene of Tn5, and the 3' terminal sequences of the octopine synthase gene (Dhaese et al., ref.15).

The procedure involves the following steps:

- in vitro inoculation of stem fragments followed by culture in vitro on callus inducing medium,

- selection for the kanamycin resistant trasformed plant cells on medium containing kanamycin,

- subsequent culture of the transformed calli on media which allow the regeneration of shoots,
- culture of these shoots on media inducing root formation.

In more detail the procedure is carried out as follows:

(1) Germinate and grow Asparagus officinalis cv. "Ruhm v. Braunschweig" in pots with vermiculite under artificial light (5000 Lux, 16 hrs/day, cool white fluorescent tubes) at 22° C. Water daily with mineral fertilizer solution (Bayfolan Bayer, 0,3 ml/l) supplemented with the micro-elements of the Murashige and Skoog medium (ref. 12).

(2) Remove young spears, 20 cm long from 10 month old seedlings. They are 2-3 mm in diameter.

From now on, all manipulations are done under aseptical conditions.

(3) Surface sterilize the spears applying the following treatment
- 70% ethanol for 2 min.
- 0,5% $HgCl_2$ in H_2O for 5 min.
- 10% commercial bleach and 1 % laboratory detergent (Decontamin) for 30 min.
- 5 rinses with sterile tap water.

(4) Cut spears in 3 cm long segments with a scalpel.

(5) Place 10 fragments vertically and upside down in a 250 ml glass top jar containing 50 ml half-strength LS medium, solidified with 0,7 % Difco Bacto agar. The fragments are inserted 5 mm in the agar medium.

(6) Grow Agrobacterium tumefaciens strain C58C1Rif^{R}pGV 2302 on an LB plate with 25 mg/l Km for 48 hrs. at 28°C. Take bacteria from the plate with a spatula and apply them to the top of the segments. Incubate for 1 month in a plant culture room (1.500 Lux, 16 hrs/day, at 24 ° C)

(7) Excise the upper 2 mm of the stem fragments with a new scalpel blade.

(8) Place 5 fragments in a 5 cm petridish containing 10 ml LS medium supplemented with 1 mg/l. BAP (6-Benzylaminopurine), 1 mg/l NAA (1-Naphtalene acetic acid) (both from a stock solution at 2 mg/ml in dimethylsulfoxide), 200 mg/l glutamine and 500 mg/l Cefotaximum (Claforan, Hoechst). Incubate for one month.

(9) Transfer calli on the same medium in a 9 cm petridish. Incubate for one month.

(10) The calli are about 1 cm in diameter. Devide the calli in pieces of approx. 5 mm diameter and transfer them in 5 cm petridishes containing LS medium with 1 mg/l BAP, 1 mg/l NAA, 200 mg/l glutamine, 250 mg/l cefotaximum, and 50 mg/l kanamycin sulfate (Sigma). Use one dish per original callus. Kanamycin sulfate was prepared as a stock solution at 50 mg/ml in distilled water and the pH was adjusted to 5,7 with HCl. The solution was filter-sterilized. The pH of the medium at the moment of its use was 5.7.
Incubate for 1 month. Growth rate of the tissue in this medium is variable and in all cases slower than on kanamycin-free medium.

(11) Transfer tissue to same medium. Take care to completely remove all necrotic parts. Incubate for one month.

(12) Use approx. 20 mg samples of the tissue to detect expression of the nopaline synthase gene.
Ground the tissue in an Eppendorf tube with a plastic rod.

(13) Centrifuge for 2 minutes.

(14) Apply 2 microliter of the supernatant on a 15 cm long Whatman 3 MM paper.

(15) Perform electrophoresis towards the anode for 90 min. at 350 V using as buffer 5 % pyridine and 0,25 % acetic acid in H_2O.

(16) Dry the paper under air current.

(17) Detect nopaline using the procedure of Otten and Schilperoort (ref. 16). The results are given in Fig. 3.

Using this procedure (steps 1 - 11), 2 independent tissue culture lines which grow vigorously on media containing 50 mg/l kanamycin sulfate were obtained. Testing for nopaline in these tissues as described (steps 12 - 17) indicated that both of them indeed produce significant levels of nopaline (see Fig. 3).

As described in example 1 steps 17 to 19, we analysed the transformed calli by the Southern blot procedure. We could detect all expected fragments between the right EcoRI site in HindIII-10 and the left HindIII site of HindIII-23. This segment spans almost the whole T-DNA. We could not detect EcoRI-35 nor HindIII-23 as internal fragments, this means that respectively the left and right border are located in these fragments. Thus, again our conclusion is that the same T-DNA is recognized in monocots and dicots.

The cells lines isolated in this way can be regenerated to shoots and subsequently whole plants can be obtained by the following procedure:

(18) Shoots regenerate spontaneously from the transformed cell lines during culture on LS medium containing 1 mg/l BAP, 1 mg/l NAA, 200 mg/l glutamine and 50 mg/ml kanamycin sulphate. Excise shoots, about 1-2 cm long, from the calli and transfer onto LS medium containing 0.1 mg/l BAP. This treatment induces outgrowth of the axillary bud present on the exylant. Transfer the cultures each 4 weeks on the same medium by dividing the plantlets into tufts with 3 or 4 shoots.

(19) Induce the formation of roots by culturing tufts with 5 or 6 shoots onto LS medium made at half strength with 1 mg/l indolebutyric acid for 14 days.

(20) Transfer onto the same medium without growth regulation for 6 weeks. The plantlets will produce roots.

(21) Remove the agar from the root system by careful rinsing with water and culture the plantlets in vermiculite under the conditions described previously (see example 1). For the first watering, the nutrient solution is supplemented with tetramethylthiuram disulfide (Pomasol Forte R, Bayer , 5 g/l) to prevent fungal infections.

Using this procedure, kanamycin resistant and nopaline containing plants were regenerated from two of the transformed asparagus plants and transferred to the greenhouse.

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CLAIMS

1. A process for preparing genetically stably transformed plant cells comprising the transformation of plant cells using Agrobacterium harbouring a hybrid Ti-plasmid vector, characterized in that the plant cells are monocotyledonous.

2. A process according to claim 1, wherein the plant cells are derived from plants belonging to the order of Liliales.

3. A process according to claim 1, wherein the plant cells are derived from plants belonging to the family of the Liliaceae.

4. A process according to claim 1, wherein the plant cells are derived from plants belonging to the family of the Amaryllidaceae.

5. A process according to claim 1, wherein the plant cells are derived from plants belonging to the genus Asparagus.

6. A process according to claim 1, wherein the plant cells are derived from the plant Asparagus officinalis.

7. A process according to claim 1, wherein the Agrobacterium strain which is used contains a hybrid Ti-plasmid harbouring a gene of interest operatively linked to a promoter suitable for expression in the host cell.

8. A process according to claim 1, wherein the Agrobacterium strain which is used contains the hybrid plasmid pGV2302.

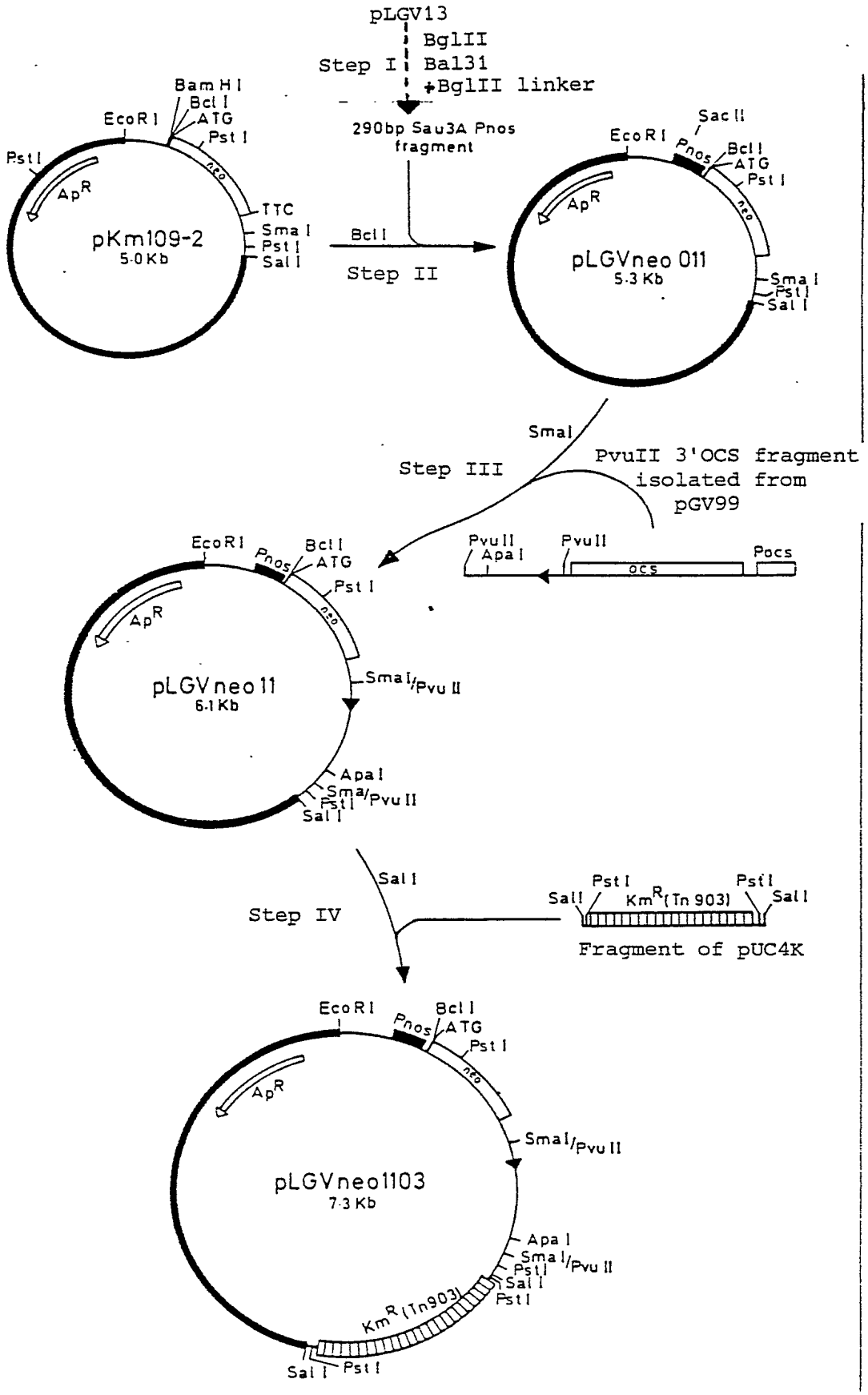
9. A process according to claim 1, wherein the Agrobacterium strain which is used contains a hybrid Ti-plasmid harbouring both a dominant selectable marker and another gene of interest operatively linked to a promoter suitable for expression in the host cell.

10. Monocotyledonous plant cells, characterized in that they are genetically stably transformed by a hybrid Ti-plasmid.

11. Monocotyledonous plant regenerated from a cell according to claim 9.

12. Agrobacterium tumefaciens $C_{58}C_1Rif^R$pGV2302 having the characteristic features of the strain deposited with the Deutsche Sammlung für Mikroorganismen, Göttingen, West Germany under the accession number DSM 3168.

Fig. 1: Construction Scheme of pLGVneo1103



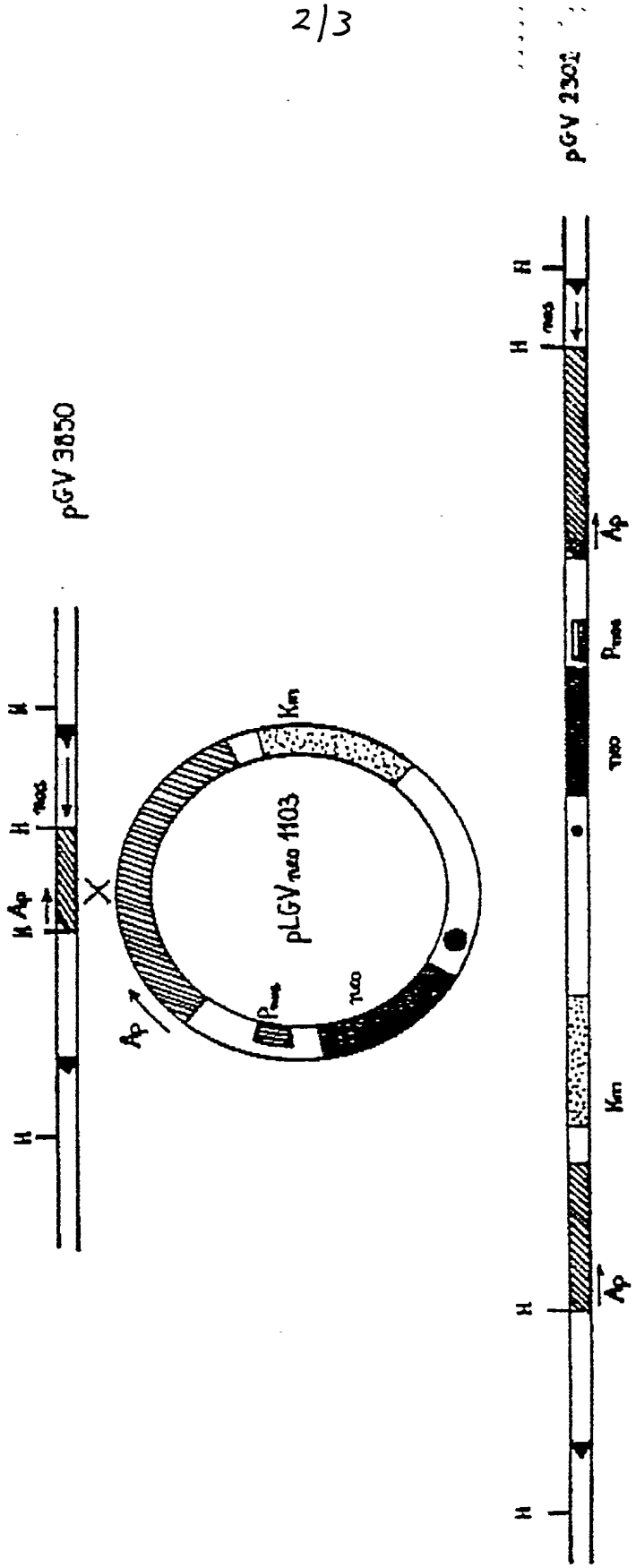
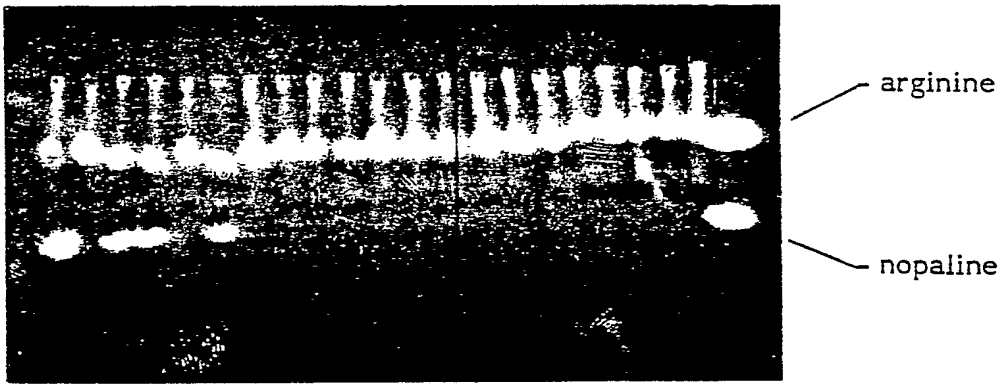


Figure 2

Fig. 3 : Dectection of nopaline in tissue extracts (steps 12 - 17.



Lane 1 : Tumor tissue of Asparagus officinalis induced by strain C58

Lane 2 : Untransformed callus tissue from Asparagus officinalis.

Lane 3-4: Two independant callus tissues of Asparagus officinalis transformed with C58C1RifpGV 2302 and grown on medium containing 50 mg/l kanamycinsulfate.

The last lane at the right end shows standard samples of 1 microgram of arginine and 1 microgram of nopaline.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 85/00726

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 1/20; A 01 H 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Nature, volume 311, 25 October 1984, no. 5988, Reading, Berks, (GB) G.M.S. Hooykaas-Van Slogteren et al.: "Expression of Ti plasmid genes in monocotyledonous plants infected with Agrobacterium tumefaciens", pages 763-764, see the whole document	1-7
Y	(cited in the application)	8-11
	--	
X	The EMBO Journal, volume 3, no. 12, 1984 J. Velten et al.:"Isolation of a dual plant promoter fragment from the Ti plasmid of Agrobacterium tumefaciens", pages 2723-2730, see page 2724, column 1, last paragraph - column 2, paragraph 1; figure 2; page 2726, column 1, paragraph 3; page 2727, column 2, paragraph 1; page 2728, column 2, lines 27-31; page 2729, column 1, lines 16-33	12
Y		8-11
	--	
P,X	The EMBO Journal, volume 3, no. 13, 1984, J.P. Hernalsteens et al.:"An Agrobacterium transformed cell culture from the	./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th April 1986	13 MAI 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	monocot asparagus officinalis", pages 3079-3041, see the whole document	1-3,5-7,10, 11
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P,X	EP, A, 0159418 (RIJKSUNIVERSITEIT LEIDEN) 30 October 1985, see the whole document	1-7,9-11
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A	Nature, volume 311, October 1984, no. 5988, Reading, Berks, (GB) M.H. Drummond:"Plant engineering: More prospects for Ti plasmids", page 706, see the whole article	9
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A	Science, volume 222, 18 November 1983, A. Caplan et al.:"Introduction of genetic material into plant cells", pages 815-821, see page 821, column 3, paragraph	1
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A	The EMBO Journal, volume 2, no. 12, 1983, P. Zambryski et al.:"Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity", pages 2143-2150,	
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A	WO, A, 83/01176 (INTERNATIONAL PLANT RESEARCH) 14 April 1983	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/EP 85/00726 (SA 11709)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/04/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0159418	30/10/85	JP-A-	60227683	12/11/85
		NL-A-	8401048	01/11/85
WO-A- 8301176	14/04/83	AU-A-	9054882	27/04/83
		EP-A-	0090033	05/10/83